Comparison of biomarker responses following one dose of benzo-a-pyrene administered to three native Australian fish species

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Manuscript received February 2011; accepted March 2011

Abstract

The Australian native fish pink snapper (*Pagrus auratus* Forster) is currently used as a bioindicator species for laboratory and field studies, but is often unavailable from hatcheries, or collected in limited numbers in the field. Consequently, mulloway (*Argyrosomus hololepidotus* Lacépède) and barramundi (*Lates calcarifer* Bloch), two Australian native fish species, were tested in an exploratory study as potential bioindicator surrogates to pink snapper. Experimental fish were i.p. injected with benzo(a)pyrene (BaP), a well known biomarker inducer in fish, at a dose of 1.0 µg/g of fish. Physiological indices i.e. condition factor (CF) and liver somatic index (LSI) and a suite of biomarkers including ethoxyresorufin-O-deethylase (EROD) activity, biliary metabolites, serum sorbitol dehydrogenase (SDH), DNA damage (Comet assay) and heat shock proteins HSP 70 were explored in the three test species. Mulloway and barramundi showed a higher response in biliary metabolite levels than pink snapper, while pink snapper showed a higher EROD induction potential relative to mulloway and barramundi. Mulloway appeared to be sensitive to hepatotoxicants, as the chemical injury sustained by the liver resulted in the release of SDH in the bloodstream of this species. All three species were significantly responsive to DNA damage. When injected with BaP, the three species showed similar response for CF, LSI and HSP 70. Initial results indicate that mulloway and barramundi are suitable surrogate bioindicator species for pink snapper in relation to exposure to BaP.

Keywords: Pink snapper, Mulloway, Barramundi, BaP, Fish biomarkers, EROD, SDH, Bile metabolites, DNA damage, Comet assay, HSP 70

Inroduction

Pink snapper (*Paragus aratus* Forster 1801) is found in warm temperate to sub-tropical waters of southern Australia (Paulin 1990), and is one of the most popular commercial and recreational fish in this region. Pink snapper may reach a maximum length of 1.3 m and weight 19.5 kg (Hutchins & Thompson 1983). Juvenile pink snapper is currently used as biological tool for evaluation of biotic response to marine pollution (Gagnon & Rawson 2009; Ranaldi & Gagnon 2010). The biochemical responsiveness of pink snapper to the common pollutants pentachlorophenate (PCP) and polychlorinated biphenyls (PCBs) has been demonstrated (Tugiyono & Gagnon 2001), validating the suitability of this species as a bioindicator of environmental contamination. Limitations of this species include its extremely slow growth rate and the unreliable availability of juveniles from hatcheries. Its biology is also lacking in information, with the biology of juvenile fish mostly unknown. When pink snapper is unavailable, a surrogate species needs to be identified. Therefore, mulloway (*Argyrosomus hololepidotus* Lacepède 1801) and barramundi (*Lates calcarifer* Bloch 1790), both native to Australia, were identified as potential alternative bioindicator species.

Mulloway occur mainly in coastal embayment and estuaries, but also occur off ocean beaches and on inshore reefs to depths of about 100 m. Small mulloway tend to remain in the more saline estuaries. These fish are effective predators, feeding upon whatever prey is available in their particular habitat (DPI & F 2003). Mulloway is available all the year round from the field, or from hatcheries where it is grown for aquaculture ventures.

Barramundi is among the largest and most important commercial fishes in Australia (Allen 1997). Barramundi are large predators which can grow up to 60 kg and 180 cm in length. Barramundi are protandric hermaphrodites, reproducing as males between 2 and 6 years of age, after which they reproduce as females (Davis 1986). This

species has a very extensive range in tropical and semi-tropical areas of Northern Australia, Papua New Guinea and Asia, and has potential to be used as a biomonitor of environmental pollution in the natural water bodies of the region (Mercurio *et al.* 2004; Codi King *et al.* 2005; Humphrey *et al.* 2007).

Polycyclic aromatic hydrocarbons (PAHs) are an important environmental threat. Some PAHs have well documented toxic, mutagenic and carcinogenic potentials that make them priority pollutants (Naes *et al.* 1999). Benzo-a-pyrene (BaP) is a well known inducer of biochemical reactions in fish, *e.g.* ethoxyresoryfin-*O*-deethylase (EROD) activity (Ramsak *et al.* 2007; Yun *et al.* 2008) and is used as a common standard to trigger biological responses under laboratory conditions. In addition, BaP is an ubiquitous anthropogenic contaminant found in urban and marine environments. Several studies have exposed fish to BaP either via the water column or through intra-peritoneal (i.p.) injection to the animal in order to measure the cellular response of the animal to the chemical (Lemaire-Gony 1995; Bonacci *et al.* 2003; Ramsak *et al.* 2007). In exploratory studies, i.p. injections are often the preferred route of exposure as it ensures that all organisms receive the same dose of toxicant, which reduces variability of the measured parameters. BaP at dose of 1.0 µg/g of fish has been shown to be high enough to trigger the biochemical responses in fish tissues with no mortality (Bakhtyar & Gagnon 2009). However, several studies have also administered much higher doses of BaP through i.p. injections in a variety of species without any mortality. For example; the juvenile European sea bass *Dicentrarchus labrax* (Lemaire-Gony 1995), the European eel *Anguilla anguilla* (Bonacci *et al.* 2003), the juvenile Arctic charr *Salvelinus alpinus* (Padrós *et al.* 2003), as well as black gobies *Gobius niger* (Ramsak *et al.* 2007), and the juvenile gilthead sea bream *Sparus auratus* (Banni *et al.* 2009) all have been the subject of biomarker studies using i.p. BaP injections.

Under laboratory conditions, juvenile pink snapper, mulloway and barramundi were injected with BaP in order to investigate their biochemical responsiveness to this model contaminant. A suite of physiological responses (condition factor: CF, liver somatic index: LSI) and biochemical markers specific to organic compounds *i.e.* EROD activity, biliary metabolites, biomarkers of general stress such as serum sorbitol dehydrogenase (SDH), heat shock proteins (HSP 70) and DNA damage (Comet assay) were measured in order to assess the potential of each species as a suitable alternative to pink snapper.

Material and Methods

Fish Maintenance

Juvenile pink snapper, mulloway and barramundi were obtained from a local hatchery (Fremantle TAFE, WA). Sexually immature fish were purposely selected for the current study as the presence of steroid hormones may influence some biomarker responses, *e.g.* EROD activity (Gagnon *et al.* 1994).

The basic procedures for tank setup followed the standard handling and water renewals from the US Environmental Protection Agency EPA 821/R-02-012 (2002). Aeration was constant in order to maintain close to 100 % saturation of oxygen in the aquarium water at all times. The seawater (salinity 29–36 ppt, pH 7.1–7.8 and temperature 20.0–22.0°C), used for the daily 50 % water change was collected from the hatchery site where the fish were obtained, so that laboratory seawater had the same physicochemical characteristics as the water in which the fish were reared. The seawater salinity was different for the barramundi exposure as this experiment was conducted at a separate time to other fish exposures.

Eight (8) fish per treatment (4 fish per aquarium, in duplicate) for pink snapper and mulloway were kept in 100 L aquaria. However, due to large size of barramundi, six (6) fish per treatment (3 fish per aquarium only, in duplicate) were kept in 100 L aquaria. Water physicochemistry (salinity, pH and temperature) was measured daily to ensure stable physicochemical parameters. Fish were fed once a day at a maintenance ratio of 1% body weight per day during the acclimation (7 days) and experimental (4 days) periods (Palace *et al.* 1996). The food pellets consisted of a commercially available fish food (Sketting pellet type Nova ME, 45% protein, 20% lipid, 18% carbohydrates, 8% moisture).

Fish Exposure

Negative control groups received no treatment as previous laboratory trials have shown that corn oil injection or anaesthesia with MS222 (3-aminobenzoic acid ethyl ester) does not alter biomarker responses (Webb & Gagnon 2002a). All fish were anaesthetised with MS222 prior to being injected with BaP diluted in 1 mL of corn oil at a dose of 1.0 µg /g fish.

The BaP dose was selected according to previous experience (Webb *et al.* 2008) using native Australian species, where EROD activity was successfully induced to significant levels. In addition, published literature reporting EROD activity induction in other fish species did lead to the selection of a dose of 1.0 µg BaP/g fish which was intraperitoneally injected to trigger biochemical responses. All fish were killed 4 days post-injection as the biomarker response has been shown to be maximal 3 to 4 days following BaP injection (Gagnon & Holdway 1998; Au *et al.* 1999; Webb *et al.* 2008).

At the end of the exposure period, blood was collected from the dorsal vein using a vacutainer. A portion of the blood was immediately used for the Comet assay while another portion was centrifuged at 3000 *g*, at 4°C for 10 minutes and supernatant serum was collected. Fish were killed by the method known as *Iki jime* (spike through the brain). Total weight and liver weight (g) were recorded on a Sartorius Basic B3100P electronic balance, and total, fork and standard lengths were measured (cm). The gills, liver and bile were collected and immediately frozen in liquid nitrogen. All samples were stored in freezer at -80°C until analysed for biomarkers.

Fish Biomarkers

Physiological indices *i.e.* condition factor (CF) and liver somatic index (LSI) and a suite of biomarkers including EROD activity, biliary metabolites, serum SDH, DNA damage (Comet assay) and heat shock proteins HSP 70 were explored in the three test species. This suite of

biomarkers of exposure and effects are relevant to urban and industrial contamination encountered in many ecotoxicological studies.

CF was calculated according to the equation 100 x (carcass weight / standard length^3), while the LSI was calculated as 100 x (liver weight / carcass weight). Carcass weight, rather than total body weight was used as it eliminates variation due to stomach content and lipid reserves.

Ethoxyresorufin- O-deethylase (EROD) Activity

Hepatic EROD activity was analysed in liver samples. Fish livers were weighed and homogenized at a ratio of 1 part of liver with 4 parts of 0.02 M HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; pH 7.5). Homogenates were centrifuged at 9000 g for 20 minutes at 4°C and the S9 microsomal fractions were collected. All samples were prepared in duplicate according to the fluorimetric assay described in Hodson *et al.* (1991) which included a standard curve with resorufin concentrations varying from 0 to 425 pmol resorufin / mL. The reaction was initiated by adding ethoxyresorufin and terminated by the addition of analytical grade methanol to stop the reaction and precipitate proteins. Resorufin standards and samples were centrifuged at 3000 *g* for 10 minutes, and the fluorescence of the supernatant read on a PerkinElmer LS-45 luminescence fluorimeter at ex/em 530/585 nm slit 10/10. The resorufin concentrations in the samples were calculated from a standard regression after correction of measurements for blank fluorescence. Proteins concentrations in microsomes were analysed according to Lowry *et al.* (1951) using bovine serum albumin as a standard (Sigma Chemicals, Australia), on a Pharmacia UV-Visible Spectrophotometer at wavelength 540 nm. The enzyme activity was normalized to total protein contents and is reported as pmol of resorufin produced per min per mg protein (pmol mg protein-^{1}min-^{1}).

Biliary Metabolites

Naphthalene, pyrene and BaP-type metabolites were determined by fixed fluorescence (FF) measurement (Lin *et al.* 1996). It was not expected to detect high levels of fluorescence at the naphthalene wavelength however, this parameter was included in the investigation in order to insure there was no significant background fluorescence at this wavelength, for any of the fish species. Bile was diluted with 50/50 HPLC grade methanol/double distilled H_2O with a ratio of 1:2000 for the naphthalene-type metabolites. Fluorescent readings were performed against a 1-naphthol standard curve at excitation/emission 290/335 nm slit 10/10. Bile samples for pyrene and BaP-type metabolites were diluted 1:500 for negative control groups. However, experimental groups were diluted with a ratio of 1:2000 for the pyrene-type metabolites and up to 1:50000 for BaP-type metabolites in order for the fluorometric measurements to fall within their respective standards range. Metabolites fluorescing at the pyrene and BaP wavelengths were measured using 1-hydroxy pyrene as a reference standard at ex/em 340/380 nm and 380/430 nm for pyrene and BaP wavelengths, respectively.

To determine protein concentrations in bile, the bile samples were diluted 40 times with double DI H_2O and samples were prepared and incubated according to the method described in Lowry *et al.* (1951). Sample absorbances were determined at wavelengths 540 nm by using a Pharmacia Biochrom 4060 spectrophotometer.

Metabolites fluorescing at the naphthalene wavelength are expressed in mg of 1-naphthol fluorescence units equivalent per mg biliary protein, and those fluorescing at the pyrene and BaP wavelengths are reported in µg of 1-OH pyrene fluorescence units equivalent per mg biliary protein.

Serum Sorbitol dehydrogenase (SDH) activity

Fresh whole blood samples were chilled on ice immediately after collection and were centrifuged at 3000 *g* for 10 minutes. The serum was collected and immediately frozen at -80°C until analysed. Serum samples for serum SDH activity were diluted (1:9) with a solution of ß-NADH/0.1M Tris Buffer pH 7.5. Samples were incubated for 10 min at room temperature to allow the reaction of keto-acids in the serum. Enzymatic activity was initiated by the addition of fructose and the SDH activity was determined by the reduction in light absorbance at 340 nm over time (Dixon *et al.* 1987) on a LKB Biochrome 4060 spectrophotometer. Results are reported in milli-International Units (mU).

DNA damage (Comet Assay)

DNA damage was determined by the single cell gel electrophoresis (Comet assay, Trevigen, USA) according to the method adapted from Singh *et al.* (1988). Fresh blood samples (2.5–3.5 µL) were mixed with chilled PBS/DMSO solution, and the mixture added in low melting agarose (LMA) in a ratio 1:5. Sample/LMA mixture was transferred onto the two wells of a comet slide (Trevigen USA). The slides were immersed overnight in chilled lysis solution (pH 10). Alkaline horizontal electrophoresis was carried out for 20 min at 25 V and 300 mA. Slides were subsequently rinsed with DI water and submersed in chilled 80% ethanol. The slides were fixed and stained by using fixation solutions and stains provided with the kit.

Comet images (~20 per sample) were taken in greyscale on a digital microscope and stored electronically. Individual cells (~100 per sample) were quantified for DNA damage using the publicly available image analysis "CASP" programme that measures the silver stained comet parameters. The integrity of the DNA molecule was expressed as the "tail moment"; high DNA damage would lead to a high 'tail' in the micro-chromatographic process and consequently, a high tail moment represents a high DNA damage (Konca *et al.* 2003).

Heat Shock Proteins (HSP 70)

Gills were used for the determination of HSP 70 as several laboratories and field experiments have informed on the relative levels of HSP 70 present in pink snapper gills under varied conditions (Webb & Gagnon 2009). Gill samples were thawed on ice, weighed and homogenised using phenyl-methyl sulphonyl fluoride (PMSF) homogenization buffer in the ratio of 1:4 w/v. Homogenate were centrifuged at 12000 *g* at 4°C for 98 min using a Heidolph DIAX 900 homogeniser and the supernatant were collected in aliquot and stored at -80°C until analysed for heat shock proteins (HSP 70). Positive

laboratory controls (heat-shocked pink snapper) were an integral part of the measurements. Concentrations of protein were determined according to Lowry *et al.*, (1951) as described in Webb & Gagnon, (2009). The supernatant containing 40 µg protein contents was mixed with Laemmli sample buffer (Bio-Rad Laboratories Pty Ltd) in a ratio 1:2, then heated at 95°C for 4 min in a waterbath. The samples were loaded in duplicate in 12% Tris Glyscine iGels wells with heat shocked standardised control samples in two outermost wells.

The gels were run for electrophoresis (225 V, 120 mA, 40 min) followed by Western transfer. The blots were blocked with 5% skim milk dissolved in Tween phosphate buffered saline and probed overnight with monoclonal (mouse) heat shock protein 70 antibody and then incubated for 2 hrs with a secondary antibody, a goat anti-mouse IgG peroxidise conjugated (Progen Biosience Australia). The blots were incubated with a chemiluminescent substrate working solution (Progen Bioscience) for 1.5 min in a dark room and images were developed on X-Ray film using 245S X-ray Processor. The images were analysed by NIH public domain image program (http://rsb.info.nih.gov/nih-image) for quantification of protein density. Heat shock protein levels are reported in pixel/µg proteins.

Data Analysis

The analysis was performed using SPSS for Windows version 16. All data were log transformed to achieve normality of distribution and homoscedasticity of variances. T-tests were used to compare between duplicates of the same treatment for example, between the two control aquaria of a species. Statistical comparisons (t-tests) of duplicate aquariums confirmed that there was no differences ($p > 0.05$ in all cases) between duplicates and consequently, the two aquariums for the same treatment were pooled for further statistical comparisons. T-tests were used to compare experimental groups to their respective negative control groups. Data was presented as means ± standard error (SEM).

Results

The physicochemical water parameters, salinity, pH and temperature, were stable throughout the acclimation and experimental (total 11 days) periods (Table 1). Fish were feeding well and were in good physical condition throughout the experiment.

The total weight and length of pink snapper and barramundi differed slightly between treatment groups (for total weight and total length p = 0.013, 0.001 and 0.048, 0.03 for pink snapper and barramundi, respectively) however, it is not expected that these differences would affect the selected biomarkers. CF was not significantly different between the treatment groups and their respective negative control groups (p = 0.57, 0.20 and 0.23 for pink snapper, mulloway and barramundi respectively). Similarly, LSI remained unchanged by exposure to BaP. None of the experimental group showed a significantly higher LSI relative to the negative control group (p = 0.62, 0.36 and 0.65 for pink snapper, mulloway and barramundi respectively; Table 2).

EROD activity was significantly induced in injected pink snapper and barramundi groups relative to their respective control groups. A significant difference (p = 0.001) was found between BaP injected pink snapper and the negative control pink snapper group. Similarly, BaP injected barramundi group was found significantly different (p = 0.005) from its non injected control group. However, injection of mulloway with 1.0 µg BaP per g of

Table 1

Summary of water parameters (means ± SEM) measured during acclimation and experimental periods.

Treatment	Salinity (ppt) Acclimation	Salinity (ppt) Treatment	pH Acclimation	pH Treatment	Temp (°C) Acclimation	Temp (°C) Treatment
Pink snapper Neg Ctrl	29.8 ± 0.26	30.2 ± 0.03	7.77 ± 0.03	7.71 ± 0.01	22.5 ± 0.13	21.8 ± 0.04
Pink snapper BaP injected	30.0 ± 0.05	30.4 ± 0.22	7.69 ± 0.02	7.70 ± 0.15	21.8 ± 0.45	22.1 ± 0.38
Mulloway Neg Ctrl	29.9 ± 0.21	30.0 ± 0.02	7.79 ± 0.03	7.76 ± 0.07	22.6 ± 0.23	21.7 ± 0.48
Mulloway BaP injected	29.7 ± 0.32	29.8 ± 0.17	7.65 ± 0.33	7.72 ± 0.13	22.0 ± 0.14	22.1 ± 0.59
Barramundi Neg Ctrl	36.9 ± 0.61	35.1 ± 0.32	7.55 ± 0.13	7.66 ± 0.16	20.9 ± 0.14	21.3 ± 0.23
Barramundi BaP injected	35.8 ± 0.21	36.9 ± 0.08	7.63 ± 0.22	7.58 ± 0.09	21.1 ± 0.33	20.3 ± 0.43

Table 2

Total weights, lengths and physiological indices (means ± SEM) of negative control and BaP-injected fish.

Treatment	Numbers (N)	Total Weight (g)	Total Length (cm)	Condition Factor (CF)	Liver Somatic Index (LSI)
Pink snapper Neg Ctrl	8	122 ± 24.7	17.3 ± 0.94	3.3 ± 0.24	1.27 ± 0.21
Pink snapper BaP injected	8	211 ± 17.6	22.6 ± 0.67	3.1 ± 0.09	1.12 ± 0.12
Mulloway Neg Ctrl	8	116 ± 04.5	22.9 ± 0.33	1.15 ± 0.07	1.23 ± 0.13
Mulloway BaP injected	9	125 ± 05.9	23.8 ± 0.42	1.25 ± 0.02	1.07 ± 0.12
Barramundi Neg Ctrl	6	408 ± 13.8	32.4 ± 0.37	1.35 ± 0.13	0.95 ± 0.06
Barramundi BaP injected	6	367 ± 12.0	31.0 ± 0.39	1.54 ± 0.03	0.89 ± 0.14

fish did not result in a significant EROD induction (p = 0.38; Fig. 1).

None of the species tested showed significant fluorescence at the naphthalene wavelength (p = 0.89, 0.64 and 0.07 for pink snapper, mulloway and barramundi respectively; Fig. 2A). Similarly, pink snapper and barramundi did not respond to the injection as 4 days post-injection, these two fish species did not produce significantly higher levels (p = 0.28 and 0.198 for pink snapper and barramundi respectively) of pyrene-type metabolites relative to their respective control groups. The injected mulloway, however, did produce significantly higher (p = 0.047) levels of pyrene-type metabolites compared to the mulloway negative control group (Fig. 2B). In all three species of fish, the BaP injected groups responded to the treatment with a significant (p < 0.001 in all cases) accumulation of metabolites fluorescing at the BaP wavelength, compared to their respective control groups (Fig. 2C).

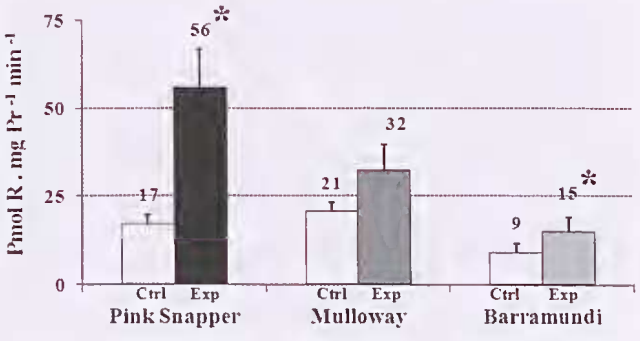

Figure 1. Hepatic EROD activity (pmol resorufin/mg protein/min) in liver of juvenile pink snapper, mulloway and barramundi injected with BaP. *indicates a significant difference (p < 0.05) from their negative control groups.

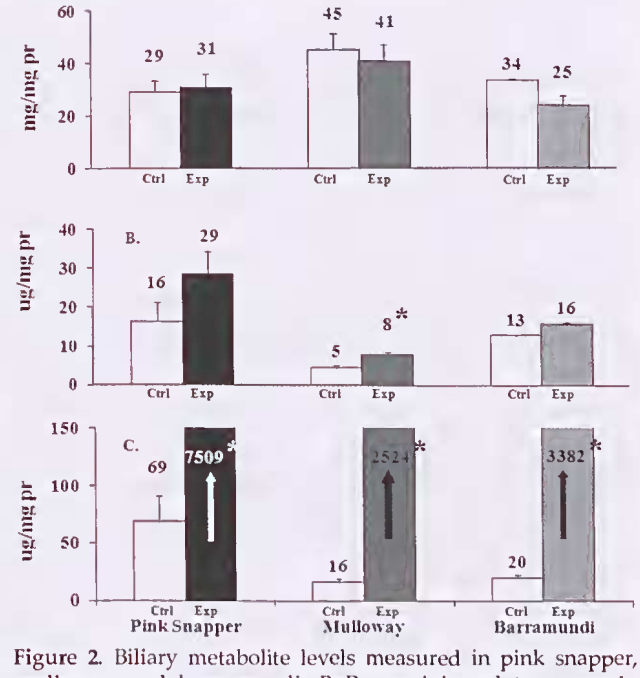

Figure 2. Biliary metabolite levels measured in pink snapper, mulloway and barramundi. BaP was injected to ascertain biochemical responsiveness and induce the presence of BaP metabolites at high levels. **A.** Naphthalene-type (mg/mg pr); **B.** Pyrene-type (µg/mg pr); and **C.** BaP-type (µg/mg pr). *For each species, indicates a significant difference (p < 0.05) from the negative control group of that species.

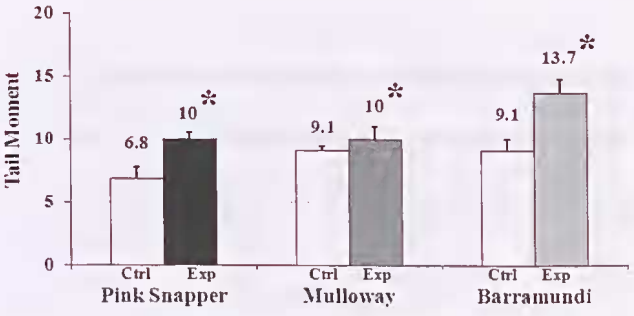

Figure 3. Serum SDH activity (mU) measured in three species of fish injected with BaP. *indicates a significant difference (p < 0.05) from negative control fish.

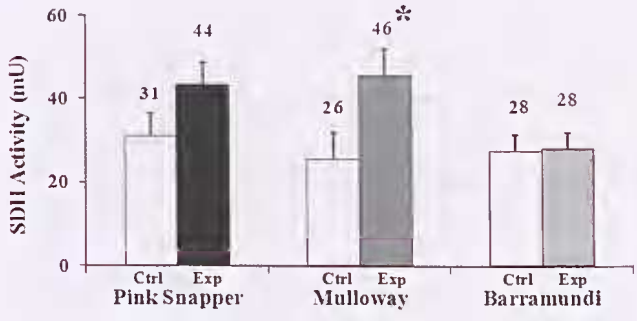

Figure 4. DNA damage (tail moment) measured in the blood of pink snapper, mulloway and barramundi injected with BaP. *Indicates a significant difference (p < 0.05) from negative control fish.

No liver damage was induced by the 4 day BaP treatment in pink snapper and barramundi, as evidenced by the lack of increase in SDH activity in the treated groups relative to the control groups (p = 0.11 and 0.99 for pink snapper and barramundi respectively). In mulloway, however, the liver SDH was released in the bloodstream and resulted in a significantly higher (p = 0.008) SDH activity in the serum of this fish species (Fig. 3).

Following injection with BaP, all three species showed a significantly higher DNA damage compared to their control groups (p < 0.001 in all cases) (Fig. 4). While the incidence of DNA damage increased by 10% in mulloway injected with BaP, the incidence of DNA damage increased by 47% and 51% in pink snapper and barramundi, respectively.

For pink snapper and mulloway, no statistical difference (p = 0.49 and 0.78, respectively) were detected in HSP 70 between the BaP treated groups (mean ± SEM 489 ± 72 and 546 ± 106 pixel µg protein^{1} for pink snapper and mulloway respectively) and their respective control groups (mean ± SEM 406 ± 48 and 553 ± 114 pixel µg protein^{1} for pink snapper and mulloway respectively). HSP 70 results for barramundi are not available due to the loss of samples during a power outrage.

Discussion

The unchanged response of physiological parameters such as LSI and CF following injection by BaP suggest the good physical condition of fish in all groups. These physiological parameters were not expected to undergo large variations as the fish were sacrificed only 96-hours post-injection.

BaP injections to pink snapper, mulloway, and barramundi caused metabolic perturbations in the three species exposed under laboratory conditions. Among the three species tested, pink snapper demonstrated significant response to BaP injection by increasing EROD induction 3-fold relative to its control group whereas, mulloway and barramundi showed lesser induction potential of 1.5 and 1.7 fold respectively, over their respective controls. However, barramundi demonstrated EROD activity by factor of 20 over controls when injected with β-naphthoflavone (Mercurio *et al.* 2004). Therefore, it is possible that the magnitude of the induction in barramundi might be greater than that achieved in our experiment (Whyte *et al.* 2000). The low EROD activity induction in mulloway and barramundi is well below the induction potential observed in other bioindicator species. For example, the EROD activity of Atlantic salmon (*Salmo salar*) can be induced over 50 times when injected to PCB126 under laboratory conditions (Smith and Gagnon, 2000), or the PCB-exposed shorthorn sculpin (*Myoxocephalus scorpius*) which had a 50-fold EROD induction relative to control fish under field conditions (Kuzyk *et al.* 2005). The most sensitive of the Australasian native species tested for EROD induction appears to be the sand flathead (*Platycephalus basseusis*) with an EROD activity of 13-fold over control fish in a field situation (Smith and Gagnon, 2000). Sand flathead are however not grown in aquaculture, and not abundant in many environments which generate the need to use alternative species. Whereas, the other tested Australasian fish species such as sixspine leatherjacket (*Meuscheuia freycineti)* and bluethroat wrasse (*Notolabrus tetricus)* have demonstrated only 2.1 and 1.3-fold EROD activity, respectively (Smith & Gagnon 2000). Amongst the species tested in the present study, pink snapper remains the most responsive species where EROD activity can be used as a sensitive biomarker of exposure to PAHs.

When mulloway and barramundi remain the most suitable or available bioindicator species, the number of fish to be collected in a field study might need to be increased over the recommended minimum of 12 fish per site (Hodson *et al.* 1991) in order to achieve acceptable statistical probability of detecting an inter-site difference. Studies show high variability of EROD activity in field studies relative to variability in lab studies (Abrahamson *et al.* 2008). Low EROD induction potentially also means that a field study needs to carefully consider the biology of the species to be collected in order to avoid confounding variables such as water temperature, stress, species difference and reproductive status, the later potentially altering EROD activity in female fish (Hodson *et al.* 1991; Goksoyr & Forlin 1992; Gagnon *et al.* 1994; Whyte *et al.* 2000; Codi *et al.* 2005; Humphrey *et al.* 2007; Kirby *et al.* 2007; Ramsak *et al.* 2007). Under field conditions, chemical analysis would also need to be carried out on the water and sediments to determine what bioavailable chemicals are present and in which concentrations, information which would complement the observed EROD induction in field-collected fish.

Not surprisingly, none of the treated groups demonstrated increased fluorescence at the naphthalene wavelength. The background fluorescence levels at the naphthalene wavelength needed nevertheless to be measured to insure that no interference would be occurring in the absence of naphthalene. The pyrene-type metabolites varied very little in the bile of all species tested. Large variations were observed in the BaP biliary metabolites of all fish species. Despite of the similar BaP dose (1.0 μg/g wet weight of fish) received by all species, the biliary metabolites of the BaP-type showed the highest response in mulloway and barramundi relative to their control groups, with BaP metabolite levels of 109-, 159- and 169-times higher than control fish for pink snapper, mulloway and barramundi respectively.

Under field conditions where fish are usually exposed to low levels petroleum contaminants, the biliary metabolite levels are not likely to be as high as in the present laboratory study. For example, sand flathead collected from the highly PAH-affected Hobson Bay (Melbourne, Victoria) showed a 4.7-times higher naphthalene levels than a reference site, while the same fish had 8.2 times the BaP-type metabolites, relative to the reference fish (Gagnon & Holdway 2002). Similarly, black bream *Acanthopagrus butcheri* collected in an urban estuary showed 4.8- and 4.4-times levels of naphthalene and BaP biliary metabolites at impacted sites relative to a reference site (Webb & Gagnon 2002b). Despite the lower response observed under field conditions, PAH biliary metabolites remains one of the most sensitive biomarker of exposure to petroleum compounds, and can provide evidence of contaminants bioavailability when chemical analysis or other biomarkers are not indicative of exposure or effects. This is supported by previous studies demonstrating that biliary PAHs show better contamination gradients than EROD activity (Hahn *et al.* 1996; Boleas *et al.* 1998; Richardson *et al.* 2001; Yun *et al.* 2008). Under extreme exposure to petroleum compounds such as an accidental oil spill, fish biliary metabolites could potentially be measured at much higher levels than under commonly occurring field conditions.

It is recognised that the time-course induction of EROD activity and of biliary metabolite induction might differ amongst the fish species. The experimental design, with a similar dose and holding conditions for all fish species *e.g.* similar water temperatures, reduced the variability potentially attributed to differing time-course events. It is therefore believed that the time-course of EROD induction and biliary metabolite excretion is comparable between species.

No liver damage resulted at 4 day post BaP treatment in pink snapper and barramundi, as evidenced by the lack of increase in serum SDH activity in the treated groups relative to their control groups. In mulloway, however, the presence of SDH activity in the bloodstream indicates the liver damage in this group (Ozretic & Krajnovic-Ozretic 1993). It has been demonstrated that SDH activity measured in the bloodstream is an early indicator of hepatocellular injury, and that serum SDH activity is detectable before physiological and histological changes are evident (Webb & Gagnon 2007). In the present study, mulloway's LSI remained unchanged probably due to the short time period allocated between injection and termination of the experiment. In addition, it can be concluded that the measured EROD activity levels in pink snapper and barramundi were a true response not influenced by hepatocellular injuries, and that the EROD activity of mulloway might have been

higher would the liver integrity have been preserved in this species. Fish livers with cellular injuries related to xenobiotic exposure are less capable of EROD induction than are non injured liver (Holdway *et al.* 1994). Mulloway appears to be the most sensitive of the species tested regarding hepatocellular toxicants.

DNA integrity was affected in all three species of fish, as seen by consistent increases in DNA damage following injection with BaP. While pink snapper and barramundi had an increase of 47% and 51% respectively in the levels of DNA damage, mulloway had a modest 10% increase showing that this former species is less sensitive to genotoxicants than pink snapper or barramundi. DNA can be affected by a variety of contaminants, especially PAHs (van der Oost *et al.* 2003), and while biliary metabolites are indicative of exposure to PAHs, DNA damage informs on biologically relevant effects experienced by the organisms.

For all species, levels of heat shock proteins HSP 70 measured in the gills remained unchanged following the 4-day treatment with BaP, which is an expected result given the short timeframe between the BaP injection and termination of the experiment. HSP 70 is also a highly variable biomarker under field conditions as several abiotic factors including exposure to heat, UV radiations, chemicals, toxic metals, poisons, air pollutants, injuries and diseases (Ali *et al.* 2003; Yoshimi *et al.* 2009) have the potential to influence HSP 70 levels making interpretation of this biomarker difficult to ascertain (Webb & Gagnon 2009).

Conclusions

Measurement of a suite of biochemical markers of exposure and effects on three species of fish indicated that mulloway and barramundi could be used as surrogate species to pink snapper for assessing the bioavailability and effects of urban and marine pollutants such as PAHs but further research is required if different classes of chemicals are to be assessed. Pink snapper showed high EROD induction potential and would be the most sensitive bioindicator if the contaminant of concern is an EROD inducer. However, mulloway and barramundi would be good alternative bioindicator species if biliary metabolites are measured but mulloway seems to be the most sensitive species to hepatotoxicants and barramundi would be the preferred bioindicator if genotoxic contaminants are present. Overall, the choice of bioindicator species will depend on the availability and ease of capture, the contaminant present in the environment and the biomarker measured. This study provides a relative responsiveness of common Australasian fish species in order to facilitate bioindicator selection.

Acknowledgements: The authors thank the staff of Curtin Aquatic Research Laboratory and the staff from Fremantle TAFE WA for the supply and handling of pink snapper, mulloway and barramundi. This study was supported by Santos Ltd, Rheochem Ltd Henderson, Western Australia, and by an Australian Research Council (ARC Linkage program) grant to MMG, and a PhD scholarship to SB.

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